ATTACHMENT 2

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PHYSICOCHEMICAL PROPERTIES OF THE PROTEOLYTIC ENZYME FROM THE LATEX OF THE MILKWEED, ASCLEPIAS SPECIOSA TORR. SOME COMPARISONS WITH OTHER PROTEASES

III. Kinetics of the Heat Inactivation of Papain, Bromelin, and Asclepain

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INTRODUCTION

The study of heat inactivation is often of considerable value in the characterization of proteolytic enzymes. This is true because in most cases these proteases appear to be inactivated at different rates at the same temperature and pH values. Also the determination of the inactivation velocity constants at different temperatures makes it possible to evaluate the critical thermal increment of the enzyme.

The thermal inactivation of crystalline trypsin, chymotrypsin, and pepsinogen is completely reversible (1), so that the denatured inactive enzymes formed by heating the solutions revert to the native condition on cooling. In the present study of the thermal characteristics of three plant proteases, papain, bromelin, and asclepain,[1] the heat inactivation could not be reversed by cooling the solutions. The rates of destruction of these enzymes do not show the great dependence on pH which pepsin exhibits (2). Near neutral pH, at constant temperatures, these plant proteases are inactivated at rates which can be described in most cases by simple equations. Differences in the state of purity do not seem sufficient to account for the individual behaviors of the enzymes.

Experimental Procedures

Enzyme Solutions. Papain.—Merck's papain powder was partially purified by first salting out the enzyme (from an aqueous extract buffered at pH 7) with $(NH_4)_2SO_4$

[1] The protease of the milkweed, *Asclepias speciosa*, whose preparation and properties have been described in the two previous papers of this series (Winnick, T., Davis, A. R., and Greenberg, D. M., *J. Gen. Physiol.*, 1940, **23**, 275, 289).

added to about half saturation, and then precipitating the redissolved enzyme with two volumes of 95 per cent alcohol. The product, washed with 70 per cent alcohol and dried in a vacuum desiccator, was more than twice as active as the original material. The solution used for the inactivation studies contained 0.33 mg. papain per ml. It was activated with dilute NaCN and then adjusted with KH_2PO_4 to pH 7.0.

Bromelin.—This enzyme was prepared from fresh pineapple fruit by the method of Willstätter, Grassmann, and Ambros (3). A solution was used which contained 1.5 mg. enzyme, per ml., activated in the same manner as papain.

Asclepain.—The solution contained 2 mg. of this enzyme per ml. of pure water. It was not buffered, and its exact pH was not known.

Method for Measuring Rates of Inactivation.—A series of small test tubes, each containing about 1.5 ml. of a given enzyme solution, were immersed in a thermostat at the desired temperature (±0.1°). The tubes were gently shaken for about a half minute and then corked, so that no water could evaporate. After varying times of heating, each tube was plunged into an ice water bath, which quickly stopped the destruction of enzyme.[2] The corked tubes were tilted and rotated horizontally to collect condensed moisture on the wall. Then the residual proteolytic activity of each solution, as well as that of the unheated enzyme solution, was measured in 1 ml. aliquots by Anson's hemoglobin method (4). The substrate, containing 2 per cent hemoglobin in about 6.6 M urea, buffered at pH 7.0, was digested for 15 minutes at a temperature of 30°.[3] The proteolysis is expressed in terms of the color value of tyrosine produced in 6 ml. of digestion mixture.

Experimental Results and Interpretation of Reaction Rates

Papain.—The rate of thermal inactivation of papain at 75, 80, and 83° was found to follow the equation of a simple first order reaction

$$2.3 \log \frac{A_0}{A} = Kt$$

where A_0 is the activity of the unheated enzyme solution, A the residual activity after heating for the time, t, and K the velocity constant. This is shown in Fig. 1. By plotting the experimental values of log A against t, straight lines were obtained, in agreement with the requirements of the first order equation.

By taking log A_0 as the intercept on the ordinate, K can be evaluated for each temperature. The constants for the different temperatures can also be calculated by substituting the experimental values of A_0, A, and t directly into the first order equation. The curves in Fig. 1 seem to depart very slightly from linearity toward the end portions, which correspond to

[2] Only a few seconds are required for the heating and cooling of these small volumes, and the time lags in starting and stopping the inactivation largely cancel each other. The time of heating was measured with a stop-watch.

[3] There is no appreciable destruction of enzyme at this temperature.

the greatest degree of inactivation. When Merck's papain was used without preliminary purification, the order of the inactivation was indeterminate.

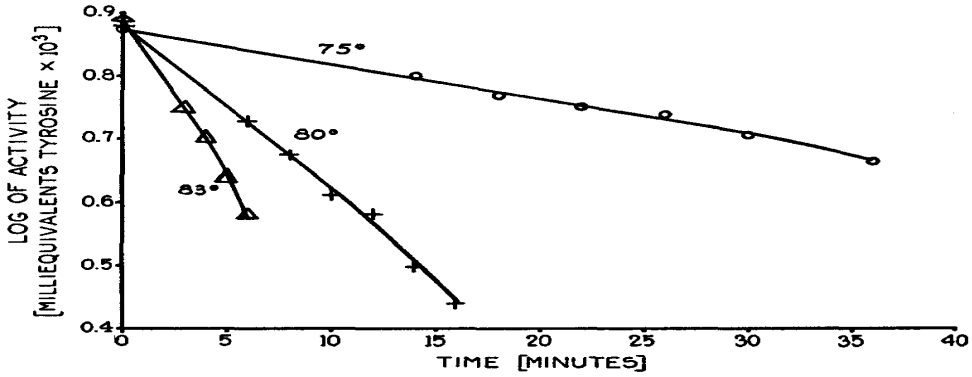

FIG. 1. Rate of heat inactivation of papain

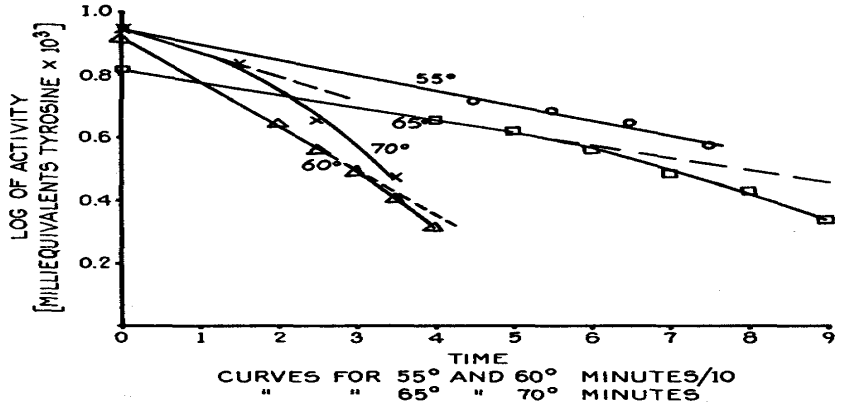

FIG. 2. Rate of heat inactivation of bromelin

Bromelin.—The rate of inactivation of this protease was found to follow the first order equation at 55°, and nearly so at 60°, but at higher temperatures the destruction of enzyme was greater than the first order equation

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required. This was shown by the gradual rise in the values of the velocity constants, which were calculated from the first order equation.

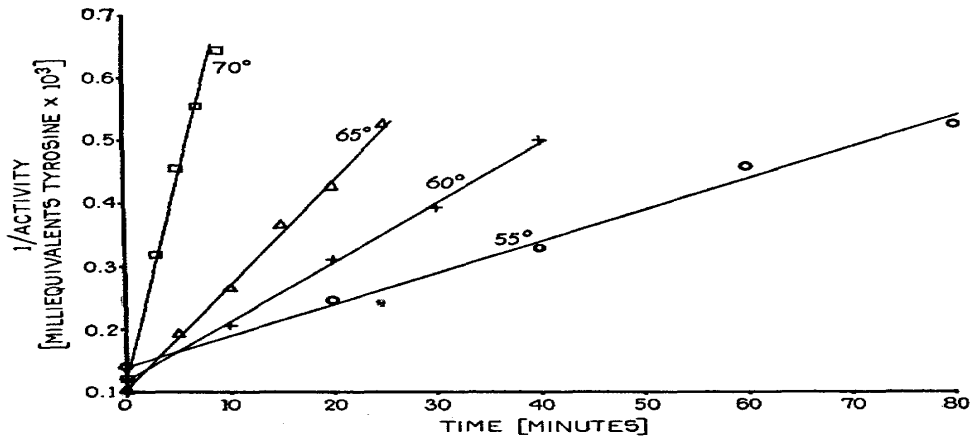

FIG. 3. Rate of heat inactivation of *Asclepias* protease

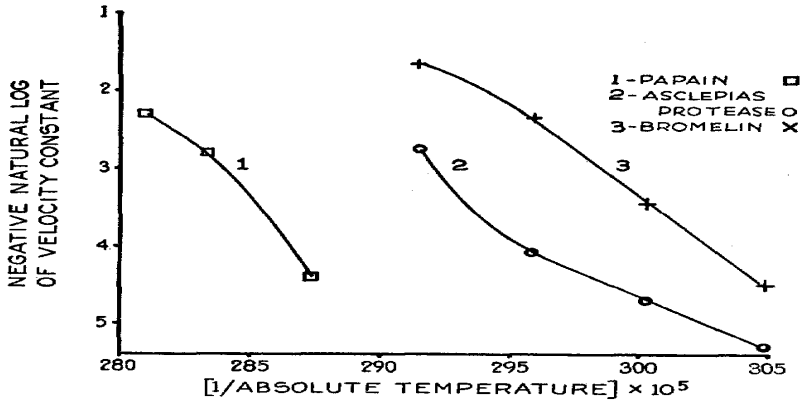

FIG. 4. Relation between velocity constants and absolute temperature

In the plots of log A against t, given in Fig. 2, the deviations from linearity are more marked than in the case of papain, and increase progressively with increase in temperature. It was necessary to use the initial linear

portions of the curves in order to evaluate graphically the velocity constants, particularly at 65 and 70°.

It was thought possible that the deviations from the first order rate were due to the inhibition of part of the active enzyme by combination of the latter with the inactive fraction. If this hypothesis were correct, one would expect a mixture of active (unheated) and completely inactivated enzyme to have less activity than a solution having the same concentration of the active enzyme alone. This test was performed, and it was found that the former solution did have slightly less activity than the solution of only the active enzyme. But this difference was too small to account for the observed deviations in the inactivation rates. It may be noted that Michaelis and Rothstein (5), in their study of the inactivation of rennet by alkali, found that the inactive enzyme did not influence the rate of destruction of the remaining active portion.

Asclepain.—This enzyme differed qualitatively from papain and bromelin in that its inactivation followed the course of a second order reaction at 55, 60, 65, and 70°. The second order equation may be written as

$$\frac{1}{A} - \frac{1}{A_0} = Kt.$$

The letters have the same significance as before.

In Fig. 3 it is seen that the plots of $1/A$ against t give straight lines in accordance with the second order equation. K was evaluated from the slopes of the lines according to the relation $\frac{d(1/A)}{dt} = K$. If the experimental data are substituted into the second order equation, it is found that the velocity constants for each temperature do not vary beyond the limits of experimental error. The data do not fit the first order equation.

Correlation of Velocity Constants

The heat inactivation of pure enzymes, such as crystalline pepsin (6), almost invariably follows the first order equation. It is interesting that the inactivation of impure papain and bromelin also follows this reaction order (at certain temperatures). The only hitherto recorded instance of second order inactivation of an enzyme is that reported by Kunitz and Northrop (7) for crystalline trypsin. Between pH 2.0 and about 9.0 the irreversible inactivation of this protease is a second order reaction. The explanation offered is that the active native protein (trypsin) hydrolyzes the denatured form with which it is in equilibrium. The investigation of the second order inactivation mechanism in the case of asclepain was not feasible, due to the impure condition of the enzyme preparation.

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The average values of the velocity constants of papain, bromelin, and asclepain are recorded in Table I. Since the first and second order constants are not strictly comparable, it is more convenient to use the half life period[4] to compare the rates of inactivation of the three enzymes. Using this criterion, it is seen that the rate of destruction of papain at 75° is about equal to that of bromelin at 55° and only about half as great as the rate for asclepain at 55°. At 70° bromelin and asclepain are inactivated more than 20 times as fast as is papain at 75°. It is clear that papain is by far the most resistant to heat inactivation, and that the thermal

TABLE I

Comparison of Thermal Characteristics of Three Plant Proteases

Enzyme	Order of reaction for heat inactivation	Temperature	Velocity constant (from curves)	Velocity constant (from equations)	Half life period (from curves)	Critical thermal increment
		degrees	$K \times 100$	$K \times 100$	*min.*	*cals. per mol*
Papain	First	75	1.23	1.26	56	
		80	6.0	6.1	11.5	75,000 (75–80°)
		83	11.0	11.2	6.3	51,000 (80–83°)
Bromelin	First	55	1.12	1.11	62	
		60	3.2	3.3	21.5	46,000 (55–60°)
		65	8.8	9.3*	7.9	45,000 (60–65°)
		70	19.2	—	2.6	36,000 (65–70°)
Asclepain	Second	55	0.51	0.50	28	
		60	0.95	0.92	13	27,000 (55–60°)
		65	1.65	1.65	6.5	25,000 (60–65°)
		70	6.4	6.3	1.9	61,000 (65–70°)

* Average of values for the 4, 5, and 6 minute preheating times.

stabilities of bromelin and asclepain (particularly between 65–70°) are not very different.

The temperature coefficients of the destruction rates are best considered in their relation to the corresponding energies of inactivation, the critical thermal increments. These latter values were calculated for the three enzymes with the aid of the van't Hoff-Arrhenius equation

$$\frac{d \ln K}{dT} = \frac{E}{RT^2}$$

which relates the reaction velocity constant, K, the absolute temperature, T, and the critical thermal increment, E. R is the gas constant.

[4] The time required for the proteolytic activity to be reduced to half its initial value.

Integrated, this equation may be written in the form

$$\ln K = -\frac{E}{1.98T} + C.$$

According to this equation, the plot of ln K against $1/T$ should give a straight line whose slope is $-E/1.98$. The plot of these variables, given in Fig. 4, shows some departure from straight lines, particularly for the curves of bromelin and asclepain at higher temperature values. This suggests that E is not constant for the whole of the temperature ranges. If the regions of 75–80° for papain, and 55–65° for bromelin and asclepain, are considered as linear, the critical increments (in calories per mol) as calculated from the slopes are: papain, 75,000; bromelin, 48,000; asclepain, 27,000.

The van't Hoff-Arrhenius equation integrated between the limits T_2 and T_1 is

$$\ln \frac{K_2}{K_1} = \frac{E(T_2 - T_1)}{1.98\,T_1T_2}.$$

The equation in this form was used to calculate E for the separate temperature intervals. The resulting values of the critical increments given in Table I are seen to correspond to those evaluated from the curves.

The critical increments for papain and bromelin are apparently of the same high order as the values which are reported in the literature for several other enzymes and substances closely related to enzymes. Asclepain (55–65°) has a somewhat lower value of E.

Steinhardt (2) and La Mer (8) have concluded that the values for the energy and entropy of enzyme denaturation and for protein denaturation generally are illusory, since the comparisons of rates at constant pH alone may be fallacious. In the case of pepsin, the customary method measures in addition to the energy of activation, the heat of dissociation of groups involved in acidic equilibria. When this factor was allowed for, the true critical increment becomes 18,300 instead of 63,500 calories for this enzyme. In the present study of inactivation, the effects of varying pH on the rate of destruction were not studied, but it seems possible that the differences in critical increments for the plant proteases are in part due to different heats of dissociation of groups in the enzyme molecules. In any event, the high critical increments, particularly those of papain and bromelin, suggest that heat inactivation involves the breaking of a number of bonds in the enzyme molecule, as is the case in protein denaturation. This agrees with the evidence for the protein nature of plant proteases.

SUMMARY

1. The rates of heat inactivation of papain, bromelin, and asclepain were determined at several different temperatures. Papain was by far the most resistant to heat.

2. The destruction of papain at 75–83° and bromelin at 55–70° followed the course of a first order reaction, except that for longer times of heating, bromelin (at 60–70°) was inactivated more rapidly than the first order equation required.

3. The rate of inactivation of asclepain at 55–70° followed the second order equation.

4. The critical thermal increments of inactivation of papain and bromelin, calculated with the van't Hoff-Arrhenius equation, were of the same high order that has been found for protein denaturation. The increment for asclepain was somewhat lower.

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